Immune Elimination of Host Fibroblasts for the Cultivation of Human Tumors Transplanted into Nude Mice

Tetsuro Okabe, Atsuko Suzuki, Nakaaki Ohsawa, Kinori Kosaka, and Toyozo Terasima

Material and Methods: Nu/Nu mice (8 to 12 weeks old) were kindly provided by Dr. Y.

ABSTRACT

We report here a useful method for the isolation and cultivation of human tumor cells in vitro from human tumors grown in nude mice. A rabbit was immunized with spleen cells obtained from nude mouse tumors. The rabbit antiserum in the presence of complement effectively killed cultured cells derived from various mouse tissues, but it was not cytotoxic to cultured cells from human tissues including tumors. When mixed cultures consisting of human tumor cells and nude mouse fibroblasts were treated with the antiserum and complement, the nude mouse fibroblasts were completely removed from the cultures, and the human tumor cells could be propagated without noticeable changes in morphological features.

Primary cultures of heterotransplanted human tumors grown in nude mice were also successfully treated, resulting in the ultimate elimination of fibroblastic cells derived from the stroma of the tumor. The functional properties of the tumor cells (production of human chorionic gonadotropin by choriocarcinoma cells and production of carcinoembryonic antigen by pancreas carcinoma cells) were also maintained after the antiserum treatment.

Introduction: There has been a feeling of frustration among many investigators trying to establish cell lines from human tumor cells. In particular, attempts to prepare pure cultures of tumor cells are frequently frustrated by the tendency of fibroblasts to grow more rapidly than do the tumor cells (6, 15).

On the other hand, the investigation of species antigens on cultured cells has been very practical in assisting in the identification of the species of origin of cultured cells. Methods for identifying species specificity of cultured cells include the fluorescent antibody technique, hemadsorption and cytolytic tests, and biochemical and chromosomal investigations (2, 4, 14). Some immunological techniques may be useful in discriminating and eliminating cells of specific antigenicity.

During studies on human tumors grown in nude mice, we developed a convenient method for the selection of tumor cells from stromal fibroblasts derived from nude mouse cells by means of a cytolytic reaction.

Materials and Methods: Animals. BALB/c background congenitally athymic female nu/nu mice (8 to 12 weeks old) were kindly provided by Dr. Y.

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2 To whom requests for reprints should be addressed.

3 T. Okabe, A. Suzuki, N. Ohsawa, and K. Kosaka. Establishment and characterization of a human leiomyosarcoma cell line, manuscript in preparation.

4 The abbreviations used are: FBS, fetal bovine serum; HCG, human chorionic gonadotropin; CEA, carcinoembryonic antigen.

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Cell Culture. Primary cultures of human tumors grown in nude mice had been prepared over the past 3 years to obtain human tumor cell lines (9) and nude mouse fibroblasts from the stromal elements associated with the tumors.

The human tumor was removed from nude mice and minced as finely as possible with small surgical scissors. Minced pieces of the tumor were usually treated with 0.25% trypsin (1:250; Difco Laboratories, Detroit, Mich.) in calcium- and magnesium-free balanced salt solution at 37° for 10 to 20 min. The treated pieces were dispersed in growth medium, distributed into several culture vessels (Falcon Plastics, Oxnard, Calif.), and incubated at 37° in a humidified atmosphere of 5% CO₂ in air. Since fibroblasts overgrew tumor cells in most of the primary cultures with ultimate loss of tumor cells, the isolation of nude mouse fibroblasts from these primary cultures was carried out with no difficulty.

Primary cultures of mammary carcinoma tissues (provided by Dr. Z. Yamazaki, The Second Department of Surgery, Faculty of Medicine, University of Tokyo) obtained directly from 3 patients were prepared, and human fibroblastic cell lines were similarly isolated.

A rabbit fibroblastic cell line was obtained from the uterus of an adult albino rabbit in the same way.

HF-BM was isolated from human bone marrow cells taken by sternal puncture with heparinized syringes.

Production of Antiserum. Spleen cells derived from nude mice were suspended in Eagle’s minimal essential medium with penicillin (100 units/ml) and streptomycin (100 µg/ml).

A rabbit was given 3 i.v. injections at 2-week intervals with 1 x 10⁶ viable spleen cells. Two weeks after the third injection, blood was taken, and serum was separated and heated at 56° for 30 min to inactivate the complement.

Cytotoxicity Tests for Titration of Antiserum. Cells used for titration of antiserum were spleen cells from nude mice and normal human lymphocytes separated by Ficol density gradient centrifugation (LSM solution; Bionetics Laboratory Products, Kensington, Md.). Spleen cell suspensions were prepared as described previously.

Complement-dependent cytolysis was determined by trypan blue exclusion tests in which 50 µl of cell suspension (3 x 10⁶ cells/ml) and 50 µl of antiserum in serial dilutions were mixed and 50 µl of a 10-fold diluted rabbit serum were added as the complement source. The mixtures were incubated at 37° for 45 min, and the surviving cells were counted following the addition of 150 µl of 0.5% trypan blue dye solution (2).

Cytotoxicity Tests for Cultured Cells. Cultured cells in the exponential phase of cell growth were used as target cells. About 1 x 10⁵ cells per small plastic dish (Falcon 3001; 35 x 10 mm) were treated with 200 µl of antiserum in serial dilutions, 200 µl of a 10-fold diluted rabbit serum as the source of complement, and 200 µl of complete growth medium. The dishes were incubated at 37° in a 5% CO₂ chamber for 45 min. The cells were dispersed with 0.25% trypsin and/or 0.02% EDTA in calcium- and magnesium-free balanced salt solution. The dispersed cells were resuspended in 100 µl of balanced salt solution, and the surviving cells were counted, following the addition of 100 µl of 0.5% trypan blue dye solution.

Growth Inhibition Tests. To measure the growth-inhibitory effects of immune cytolyis, survival of cells in the exponential phase of growth was assayed by the cloning technique of Puck et al. (10).

Selective Removal of Mouse Fibroblasts in Mixed Culture. Mouse fibroblasts (Nu-R) and human tumor cells (T3M-1) were seeded at the same density of 3 x 10⁶ cells/dish (35 x 10 mm) simultaneously. The dishes of mixed culture were incubated for 3 days when the total cell number reached about 1 x 10⁶ cells/dish. Serially diluted antisera and complement were added to each dish, and the dishes were incubated for 2 days without medium renewal. The number of residual mouse fibroblasts in the posttreatment cultures was counted and photographed under a phase-contrast microscope (Nikon, MD; Nippon Kogaku KK, Tokyo, Japan).

Selective removal was also assayed by the use of prelabeled mouse fibroblasts. Nu-OTUK cells were seeded and incubated in 20 ml of thymidine-free F-10 with [³H]thymidine (0.25 µCi/ml; 43 Ci/mmol; Radiochemical Centre, Amersham, England), 10% FBS, penicillin (100 units/ml), and streptomycin (100 µg/ml) in a plastic flask (75 sq cm) at 37° in a humidified 5% CO₂ chamber for 36 hr. After being labeled, the dishes were washed with Medium F-10, and the cells were harvested with 0.25% trypsin and seeded at the same density of 1 x 10⁶ cells/dish (60 x 15 mm). Twelve hr after seeding of Nu-OTUK cells, the same number of T3M-1 cells was plated on the Nu-OTUK cells in the same dishes. After 12 hr incubation of the mixed culture, serially diluted antiserum and complement were added to each dish, and the dishes were incubated for 1 hr. Then all the dishes were washed 3 times with medium F-10 and incubated in complete growth medium for 24 hr.

All the dishes were washed twice with Dulbecco’s phosphate-buffered saline, the cells were harvested with 0.02% EDTA in calcium- and magnesium-free balanced salt solution, and DNA was precipitated by adding trichloroacetic acid to a final concentration of 5%. The precipitates were dissolved in 2 ml of Protosol (New England Nuclear, Boston, Mass.) and counted [scintillation counter with a solution of Aquasol-2 (New England Nuclear)]. The assay was done in duplicate.

Effect of Antiserum and Complement on Functional Markers of Tumor Cells. The choriocarcinoma cultures of the ninth passage which had not been treated with the antiserum were used. The cultures contained a considerable number of fibroblastic cells. The cells were plated at the same density in 12 plastic dishes (35 x 10 mm) and were treated with serial dilutions of the antisera and complement. Two days later, the cultured media were harvested and stored at −20° for HCG measurements. The cultures were also harvested simultaneously for counting the number of cells. HCG was assayed by the use of a HCG radioimmunoassay kit (Commissariat a l’Energie Atomique, Gif-sur-Yvette, France) (12).

The pancreas carcinoma cultures of the second passages without the antiserum treatment were also used. The cells were plated at the same density in 12 dishes (35 x 10 mm) and treated with the antiserum in the same manner. The cultured media were also harvested after 2 days of treatment for CEA measurements, and the cells were also harvested for cell counting. CEA was assayed by the use of a CEA radioimmunoassay kit (Dinabot Radiosotope Laboratory, Tokyo, Japan) (7).

Chromosomal Studies. For chromosomal preparations, the cultured cells in the exponential growth phase were treated with Colcemid (0.2 µg/ml; Grand Island Biological Co., Grand Island, N. Y.) for 2 to 3 hr at 37° and then with hypotonic KCl solution (0.5%) for 20 min. The cells were fixed in methanol:
acetic acid (3:1). After 3 changes of fixative, the cells were
dried onto wet slides, dried over an alcohol lamp, and stained
with conventional Giemsa solution to count the chromosomes.

Karyotypic analysis revealed that Nu-R, Nu-OTUK, Nu-MBL,
and Nu-CHORIO cell lines had murine chromosomes.

RESULTS

Potency of Antiserum. The antiserum obtained by immuniz-
ing a rabbit with nude mouse spleen cells was detected by the
cytotoxicity test. The antiserum titer for 50% cytolysis was 1:
780. Human lymphocytes were not killed with the antiserum
and complement. We could not detect any cytotoxic activity
with normal rabbit serum alone.

Cytotoxicity Tests for Nude Mouse Fibroblasts (Nu-R).
Cytotoxicity tests were performed on monolayers of Nu-R cells.
The cells were incubated at a density of \(3 \times 10^4\) cells/dish
(Falcon 3001; 35 x 10 mm) and cultured for 3 days. At the
time of the tests, the cell density was about \(1 \times 10^5\) cells/dish.
Monolayer cultures were added with serially diluted antisera
and complement and then incubated at 37° for 45 min. As
shown in Chart 1, the cells showed a high sensitivity to immune
cytolysis. The titer for 50% cytolysis was 1:390. This result
indicates that the cells bear surface antigens in sufficient
amounts to be affected by the antibody and complement. This
conclusion was confirmed by the inhibition test for colony
formation.

Specificity of Antiserum. The species specificity of antise-
rum was examined with cells from various species in the same
manner as described above. Nu-R, Nu-OTUK, Nu-MBL, Nu-
CHORIO, 3T3-B, Y-1, and Sarcoma 180 cell lines, which were
derived from mouse tissues, showed a high sensitivity to im-
une cytolysis (Chart 2). Conversely, human cell lines such as
HT-73, T3M-1, T3M-2, NB-1, GOTO, HF-MAM-1, HF-MAM-2,
HF-MAM-3, and HF-BM were not killed, even with a 3-fold
diluted antiserum and complement. FUR was not sensitive to
immune cytolysis. It was therefore concluded that there was a
difference in the specificity of the complement-fixing antibody
for mouse and human cells.

Time Response of Nu-R Cells Treated with Fixed Doses of
Antiserum and Complement. The incubation time required for
killing Nu-R cells was examined as described above. Chart 3
shows the time response of Nu-R cells at a density of \(1 \times 10^5\)
cells/dish (35 x 10 mm) to 2 fixed doses of the antiserum and
complement. The antiserum diluted 1:192 showed maximal
killing of the target cells with a 2-hr exposure. However, with
the 1:384 dilution of antiserum, 4 hr of treatment were required
to kill all the cells. The result indicates that the cytotoxic effect
increases with incubation time and that limiting dilutions of
antiserum require a longer time beyond the conventional incu-
bation period to kill all the target cells.

Effect of Cell Density on Immune Cytolysis. Cytotoxic titra-
tion of antiserum was performed against different densities of
Nu-R cells in a monolayer at a fixed 45-min treatment time in
the same manner. Chart 4 shows that the extent of cytolysis
depends upon cell density and concentration of antiserum, i.e.,
the more dense the culture, the more antibody is needed for
immune cytolysis. Even confluent cultures (more than \(4 \times 10^5\)
cells/dish) responded to the 1:12 dilution of antiserum with
ultimate cytolysis of 97% of the target cells. The results indicate
that a large amount of antiserum is necessary to kill all the
target cells in a dense culture.
Growth Inhibition Tests. Nu-R cells were seeded at a density of 100 cells/dish (60 x 15 mm) and cultivated for 3 days when the sparsely populated colonies were growing. Serially diluted antisera and complement were added to each dish, and the dishes were incubated for 4 days without medium renewal. After fixation, the cultures were stained in Giemsa solution, and survival was determined by counting the colonies developed in the dishes. The efficiency of colony formation was 68% in untreated control cultures. Chart 5 shows the relationship between survival and the antiserum concentration. The titer of the antiserum for 50% inhibition of colony formation was 1:43,000.

To confirm the morphological observations described above, mixed cultures of T3M-1 cells and [3H]thymidine-labeled Nu-OTUK cells were treated with the antiserum and complement. After 24 hr, the cells in the posttreatment cultures were harvested, and the radioactivity was measured. No radioactivity was detected in the cultures treated even with a 64-fold diluted antisera and complement (Chart 7A). The relationship between the cell number and the radioactivity of the [3H]thymidine-labeled Nu-OTUK cells is also shown (Chart 7).

Cultivation of Human Tumors Transplanted into Nude Mice. A primary culture of a human liposarcoma grown in nude mice was prepared and treated with a 30-fold diluted antiserum and complement. The culture had become extremely crowded with the tumor cells and mouse fibroblasts by the time of treatment, and the mouse fibroblasts reappeared after the initial treatment. The cells in this mixed culture were subcultured at 1:3 dilution after the initial treatment, and the subculture was treated in the same manner before it became crowded. No fibroblasts reappeared after the second treatment. The tumor cells had been subcultured to passage 39. Primary cultures of a human renal carcinoma, a human pancreas carcinoma, and a human melanoma were also treated with a 30-fold diluted or a 45-fold diluted antiserum and complement before they became crowded. Mouse fibroblasts did not reappear after the initial single treatment in these posttreatment cultures. We obtained the melanoma cells of passage 18, the renal carcinoma cells of passage 2, and the CEA-producing pancreas carcinoma cells of passage 27. A primary culture of a human ependymoma had become confluent prior to the antiserum treatment. The cells in the mixed culture were subcultured at 1:3 dilution after the initial treatment with the antiserum, and the subculture was treated with a 30-fold diluted antiserum and complement. The number of T3M-1 cells was the same in all the dishes examined and was 1.6 x 10^5 cells/dish. In a culture treated with a 30-fold diluted antiserum and complement for 10 min, cytolysis of fibroblasts was observed (Fig. 1, a and b). Three days after the treatment, T3M-1 cells were found to grow but fibroblasts were no longer discernible (Fig. 1c).

Selectivity Removal of Mouse Fibroblasts in Mixed Culture. By using a mixed culture of human tumor cells (T3M-1) and mouse fibroblasts (Nu-R), the capability of the antiserum to remove mouse cells selectively was tested. Two days after the antiserum treatment, the number of residual mouse fibroblasts was counted by a phase-contrast microscope in the posttreatment cultures; 1000 cells were counted, and the number of residual fibroblasts is shown in Chart 6. Mouse fibroblasts did not reappear in the cultures treated even with a 192-fold diluted antisera and complement. The number of T3M-1 cells was the same in all the dishes examined and was 1.6 x 10^5 cells/dish. In a culture treated with a 30-fold diluted antiserum and complement for 10 min, cytolysis of fibroblasts was observed (Fig. 1, a and b). Three days after the treatment, T3M-1 cells were found to grow but fibroblasts were no longer discernible (Fig. 1c).
antiserum and complement twice at 3-day intervals. No fibroblasts reappeared in the culture after the third treatment. The tumor cells have been subcultured to passage 5. The more crowded culture was treated with a 45-fold diluted antiserum and complement, but the fibroblasts reappeared. The cells in the posttreatment culture were subcultured at 1:3 dilution, and the subculture was treated twice at 2-day intervals in the same manner. Mouse fibroblasts did not reappear in the culture after the third treatment. The less crowded culture was treated with a 45-fold diluted antiserum and complement, resulting in the ultimate elimination of all the mouse fibroblasts with the initial single treatment. The choriocarcinoma cells have been subcultured to passage 56 and produce HCG. A primary culture of colony-stimulating factor producing thyroid carcinoma was also treated in the same manner with the ultimate elimination of all the mouse fibroblasts. The tumor cells have been subcultured to passage 30.

The choriocarcinoma cultures and the pancreas carcinoma cultures were also treated with serially diluted antiserum and complement to see whether their functional properties (HCG production by the choriocarcinoma cells and CEA production by the pancreas carcinoma cells) were maintained after the antiserum treatment. The total cell numbers and HCG or CEA production by $1 \times 10^5$ cells for 48 hr are shown in Table 1.

### Table 1

<table>
<thead>
<tr>
<th>Antiserum Dilution</th>
<th>Before treatment</th>
<th>After treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell no. $\times 10^5$ cells/48 hr</td>
<td>ng/10^5 cells/48 hr</td>
</tr>
<tr>
<td>HCG Non-treated control</td>
<td>1.12</td>
<td>24.8</td>
</tr>
<tr>
<td>1:4</td>
<td>1.12</td>
<td>25.9</td>
</tr>
<tr>
<td>1:16</td>
<td>1.12</td>
<td>24.6</td>
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<tr>
<td>1:64</td>
<td>1.12</td>
<td>27.0</td>
</tr>
<tr>
<td>1:256</td>
<td>1.12</td>
<td>25.4</td>
</tr>
<tr>
<td>CEA Non-treated control</td>
<td>1.01</td>
<td>31.3</td>
</tr>
<tr>
<td>1:4</td>
<td>1.01</td>
<td>27.7</td>
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<td>1.01</td>
<td>30.1</td>
</tr>
<tr>
<td>1:64</td>
<td>1.01</td>
<td>29.3</td>
</tr>
<tr>
<td>1:256</td>
<td>1.01</td>
<td>28.9</td>
</tr>
</tbody>
</table>
About 140% HCG per 1 x 10^6 cells was produced in choriocarcinoma cultures treated with a 16-fold diluted antiserum and complement, and no fibroblasts were discernible in these cultures. About 170% CEA per 1 x 10^6 cells for 48 hr was produced in the pancreas carcinoma cultures treated with a 16-fold diluted antiserum and complement. Fibroblasts did not reappear in the cultures.

The results indicate that the treatment of primary cultures of human tumors producing a marker (HCG or CEA) allowed the long-term passage of a population of cells that appeared human morphologically and continued to produce the tumor marker at a high rate per cell without becoming overgrown with non-marker-producing cells (fibroblasts).

**DISCUSSION**

Both human and animal tumors have been successfully transplanted into athymic nude mice and maintained by serial passages. Several interesting human tumors have been maintained in nude mice, and preservation of various characteristics of the original tumors has been confirmed by many researchers (8). In attempts to isolate human tumor cell lines, the use of these heterotransplanted tumors is remarkably convenient for repetition of primary cultures. However, it is very difficult to obtain pure cultures of tumor cells from these heterotransplanted tumors. The desired cells are tumor cells, but the cells usually obtained are host mouse fibroblasts from the connective tissue elements associated with the tumor.

In this communication, immune elimination of nude mouse fibroblasts, when properly timed, was found to be a useful method to remove the contaminating host fibroblasts from primary cultures of heterotransplanted human tumors grown in nude mice. In general, the antiserum titers required to remove host fibroblasts from the primary cultures were 1:30 to 1:45. Fibroblasts did not reappear in the low-cell-density primary cultures after the initial treatment. However, in the high-cell-density primary cultures, in which the tumor cells and fibroblasts contact closely and adhered to each other, the fibroblasts adhering to the tumor cells or to each other tended to reappear in the posttreatment cultures. Therefore, the cells in the posttreatment cultures should be subcultured after the initial treatment of the primary culture. After subcultivation, the second or the third treatment at 2- to 3-day intervals may be required before the cultures become crowded to remove all the contaminating mouse cells in these cultures. Whether removal of fibroblasts diminishes or enhances the growth of the human tumor cells is an important problem. It was technically difficult to obtain quantitative data on that problem in the primary culture. However, we have the impression that the growth of human tumor cells was not influenced by the removal of mouse fibroblasts in our 7 primary cultures. This may be supported by our observation that the number of the tumor cells (T3M-1) was the same in all the posttreatment cultures independent of the presence of the residual fibroblasts (Nu-R). Consequently, it is recommended that the primary cultures be treated as soon as the tumor cells attach to grow and before they become crowded with host fibroblasts. The antiserum treatment of primary cultures of human tumors producing a marker (HCG or CEA) allowed the long-term passage of a population of cells that appeared human morphologically and had human chromosomes and continued to produce the tumor marker at a high rate per cell without becoming overgrown with non-marker-producing cells (fibroblasts) as do untreated cultures.

The new method described here, if tailored to the system being studied, will surely prove effective and convenient in discriminating and separating human tumor cells from contaminating host fibroblasts derived from the stroma of the heterotransplanted human tumor, thereby facilitating the isolation of human tumor cell lines (11).

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